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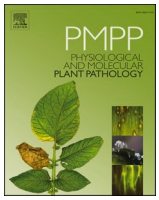

Elucidating the role of fly ash in root-knot nematode (*Meloidogyne incognita*) suppression on okra (*Abelmoschus esculentus* L.): Insights into cellular viability and host defence system

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ABSTRACT

In the present era of climatic shift, due to many natural and anthropogenic activities, it is imperative to use eco-friendly products to improve the soil quality and production of food crops, including okra (*Abelmoschus esculentus* L. Moench). As chemical nematicides are damaging to the environment, there is a need for developing alternate eco-friendly measures for the management of root-knot nematodes (*Meloidogyne* spp.) which cause major losses to vegetable crops globally. In this regard, a pot experiment was performed under optimal environmental conditions to study the nematicidal properties of fly ash (FA) on okra. Various grades of fly ash were applied in pots containing autoclaved soil before seed sowing. Results indicated that the application of 20% FA level (80:20 w/w field soil: fly ash) was found most beneficial for soil health, improving growth and yield traits, physio-biochemical attributes, and antioxidant properties of okra by alleviating the harmful effects of root-knot nematode (*Meloidogyne incognita* Kofoid and White, 1919; Chitwood, 1949) compared to other treatments. Stomatal attributes of okra also improved at 20% FA level, as demonstrated by scanning electron microscopy. Galls, egg mass formation and reproduction factor of *M. incognita* was significantly reduced at 20% FA level. Moreover, laser confocal microscopy combined with scanning electron microscopy revealed that 20% FA application enhanced root cell viability and detoxifies the accumulation of reactive oxygen species.

1. Introduction

Root-knot nematodes (*Meloidogyne* spp.) are the principal category of plant-parasitic nematodes infecting an extensive range of economically important crops all over the world [1]. More than 100 root-knot nematode species have been reported globally [2]. These hidden stressors significantly reduce the growth, yield and quality of okra all over the tropical and sub-tropical regions [3]. Infestations of root-knot nematodes are easily identified by the presence of "galls" or "knots" at the site of root feeding [4]. These nematodes result in a yield loss of about 19.6% in crops in vegetable crops throughout India [5]. Environmental scientists have been continuously alarming that soil contamination through nematicides is growing rapidly. Therefore, keeping in view human health risks and the environmental problems, it is not appropriate to suggest chemical nematicides for nematode management. Therefore, eco-friendly and feasible methods are needed for the efficient management of root-knot nematodes. Fly ash has been recommended by various researchers [6,7] for the management of these nematodes.

Okra (*Abelmoschus esculentus* L. Moench) belonging to the family Malvaceae, is an economically important biannual crop which arose from the tropics of Afro-Asian countries and is grown in hot regions [8]. It is cultivated in various parts of the world including India and represents the important plant source of proteins, lipids, carbohydrates etc. and some important elements present like Ca, Mg, Fe, Mn, K, Na and Zn. Vitamins like A and B are also present [9]. A number of biotic and abiotic factors are there which affect the crop production but root-knot

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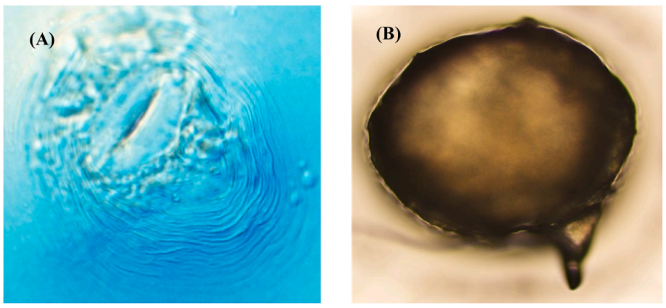


Fig. 1. Perennial pattern of *M. incognita* (A), mature female *M. incognita* (B) under stereomicroscope.

Table 1
Physiochemical characteristics of soil, fly ash, and 20% fly ash amended soil.

Parameters	FS	FA	FS + FA
Shape	Flaky	Spherical	Mixed
Texture	Sandy loam	Silty	Sandy silt loam
pH	7.34	8.21	7.57
EC (μ mhos cm^{-1})	258.22 843.56	456.87	
Porosity (%)	30.37	60.71	39.11
Water holding capacity (%)	37.22	56.88	49.53
N (%)	2.16	–	1.64
P (%)	2.66	0.048	2.21
K (%)	20.44	13.88	26.51
Calcium (mg L^{-1})	15.01	17.11	29.45
Sodium (mg L^{-1})	9.65	12.77	16.87
Chloride (mg L^{-1})	18.33	13.75	26.66
Carbonate (mg L^{-1})	71.08	62.65	89.34
Bicarbonate (mg L^{-1})	13.33	11.99	22.67
Sulphate (mg L^{-1})	14.96	22.23	28.87

nematodes are considered obscure menace to okra [10]. Hussain et al. [11,12] also found the ill effects of root-knot nematodes on okra and cause enormous yield losses. Sasser [13] reported an annual loss of about 22% in okra by plant-parasitic nematodes.

Fly ash is a coal combustion product of thermal power plants, and poses a great threat to the environment, if not managed properly [14]. The annual production of fly ash in India during the year 2021–2022 is 270.82 million tonnes, and its utilization is only 259.86 million tonnes [15], so there is a need for proper disposal of the un-utilized fly ash. Fly ash contains large amounts of macro and micronutrients for plant growth like phosphorus, potassium, calcium, sulphur, magnesium, iron, manganese, copper, molybdenum, nickel, boran etc. [16,17]. Soil fertility can be improved by the application of fly ash as it contains beneficial elements [18]. Also, there is an increase in pH, porosity, water holding capacity, and carbon content of soil [19]. At higher doses, fly ash adversely affects soil and crops as it also contains heavy metals such as lead, nickel, arsenic, chromium, cadmium etc. [20]. Fly ash is one of the economical waste material available that has great capacity to improve deteriorated soil in agriculture and forestry at lower concentration as manifested by Refs. [16,21]. Fly ash opposes the growth and reproduction of root-knot nematodes [22] by amplifying the pH of soil. The ability of fly ash as nematicide in India has been reported by various researchers like [23,24]. So, due to the presence of macro and micro elements, fly ash can be used for agricultural purposes, and it may solve many problems like utilization of waste, management of root-knot nematodes and plant growth promotion. So, taking into consideration the above mentioned points, current study was aimed to examine the effects of FA on the growth, physio-biochemical, and antioxidant properties of okra, as well as nematicidal properties of FA.

2. Materials and methods

2.1. Experimental layout

An experiment was performed under natural state, in a randomized factorial design (in 25 × 25 cm earthen pots) using autoclaved soil with an average temperature of 37 °C to study the effect of fly ash on okra at various concentrations viz. 5%, 10%, 15%, 20%, 25% fly ash (5 g, 10 g, 15 g, 20 g, 25 g fly ash kg^{-1} soil) at Botany department, Aligarh Muslim University, India. Fly ash was gathered from thermal power plant, Kasimpur, situated 18 km away from Aligarh Muslim University, India. Soil was collected from farm field and put in an autoclave for 20 min at 15lb. Okra seeds were surface sterilized with 0.1% $HgCl_2$ solution for 4 min before being cleaned with double distilled water (DDW) to remove excess $HgCl_2$ and then sown in the first week of May. The pots were filled

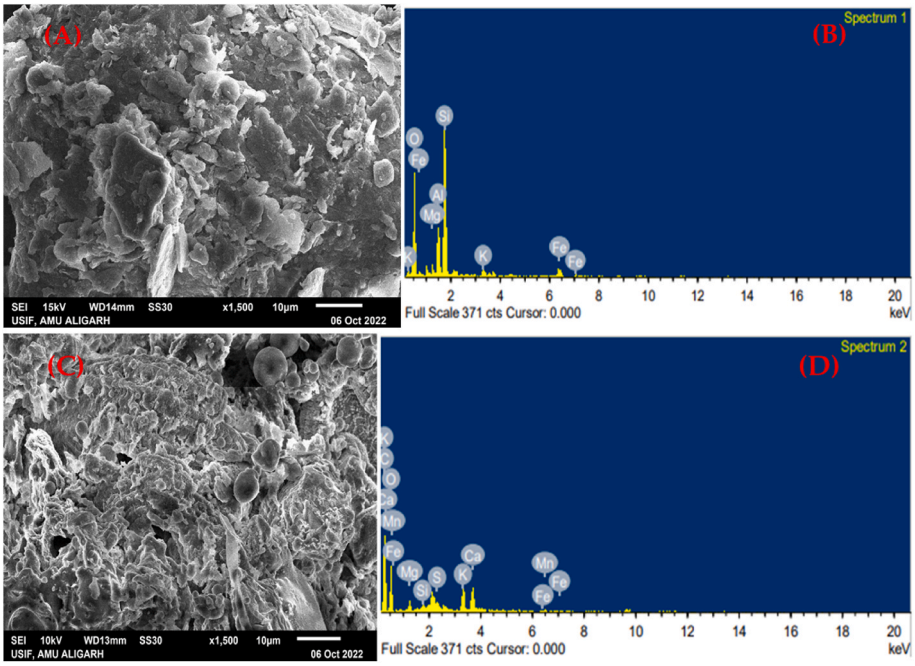


Fig. 2. SEM and EDX profiling of soil ultrastructure (A), soil chemical nature (B), FA ultrastructure (C), FA chemical nature (D).

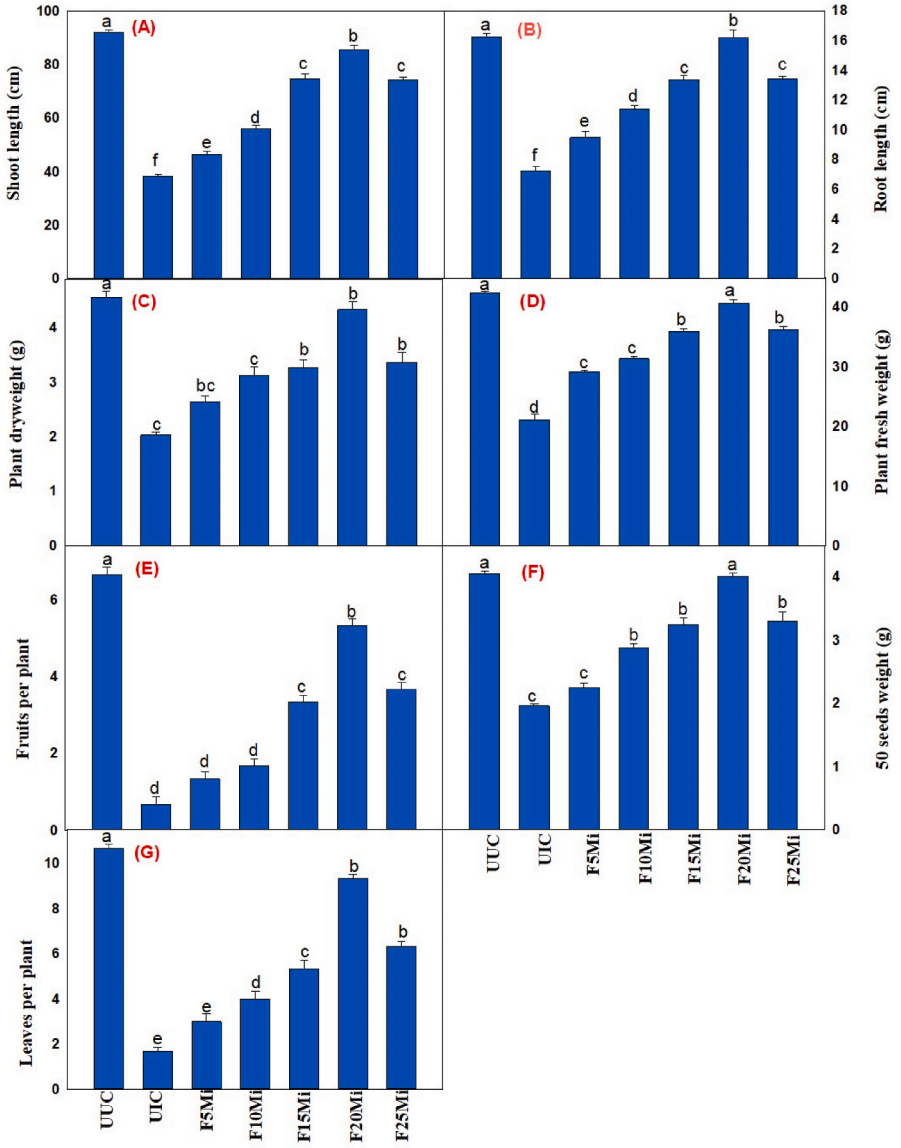


Fig. 3. Effect of soil applied FA on shoot (A) and root (B) length, plant dry (C) and fresh (D) weight, fruits per plant (E), 50 seeds weight (F), leaves per plant (G) of okra under the influence of *M. incognita*. Data represents mean of 3 replicates (n = 3). Standard errors (±SE) represented by vertical bars. Means with different letters above the bars are significantly different at $P \leq 0.05$.

with field soil (FS) and fly ash (FA), formulating various treatments as untreated uninoculated control (UUC) = 100g FS+0g FA; untreated inoculated control (UIC) = 100g FS+0g FA + *M. incognita* J2 @2000; F5Mi = 95g FS+5g FA + *M. incognita* J_2 @2000; F10Mi = 90g FS+10g FA + *M. incognita* J_2 @2000; F15Mi = 85g FS+15g FA + *M. incognita* J_2 @2000; F20Mi = 80g FS+20g FA + *M. incognita* J_2 @2000; F25Mi = 75g FS+25g FA + *M. incognita* J_2 @2000. Where F5Mi-F25Mi are various concentrations of fly ash with *M. incognita* second stage juvenile, J_2 @2000. Three replicates were kept for each treatment and each replicate contained three plants per pot. To study the growth, physio-biochemical, antioxidant, and microscopic parameters sampling was done 50 DAS (days after sowing). At harvest (80 DAS), yield and quality characteristics were studied. Upper canopy leaf samples (five from each treatment) were taken to evaluate various parameters.

2.2. Physiochemical characteristics of soil and fly ash

Jackson [25] and Rayment and Higginson [26] methods were practiced to determine the pH and electrical conductivity of fly ash and soil. Scanning electron microscope (SEM) along with Energy dispersive X-ray (EDX), using Ashfaque and Inam [27] method was used for determination of nutrients and metals in fly ash and soil. By traditional feel method i.e. rubbing fly ash or soil between fingers and thumb, texture was determined.

2.3. Inoculum preparation

Nematode inoculum was prepared according to the procedure of Khan and Siddiqui [23]. *M. incognita* species was maintained on the brinjal plants grown in the Nematology lab at Aligarh Muslim University. Egg masses were picked from the highly infected roots of egg plants by using sterilized forceps for the purpose of extraction of J2 juveniles. Egg masses were then rinsed with distilled water and placed in a tissue paper-lined sieve (9 cm diameter with 1 mm pore size). The sieves were positioned in Petri dishes with distilled water deep enough to contact the egg masses. The set-up was then incubated at 25 ± 1 °C. J2 juveniles were collected after every 24 h. After that, fresh water was added, and the procedure was repeated. An average of five counts was taken to

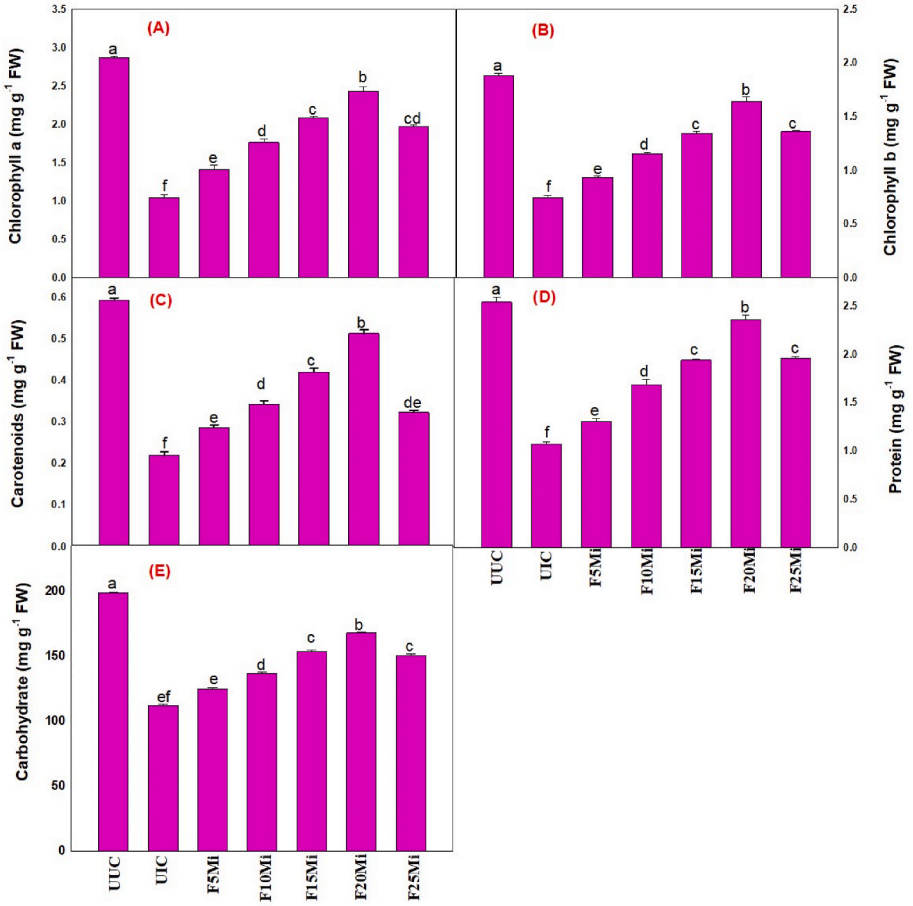


Fig. 4. Effect of different FA levels added to soil on the photosynthetic pigments (A, B, C), protein (D), and carbohydrate (E) content of okra under nematode stress. Data represents mean of three replicates (n = 3). Standard errors (±SE) denoted by vertical bars. Different letters indicate significant differences between treatments at $p \leq 0.05$.

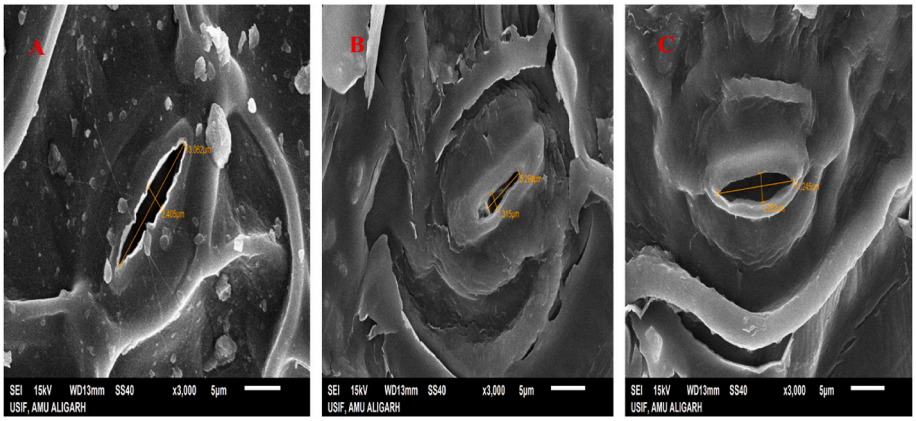


Fig. 5. Scanning electron micrograph of okra leaves, showing the stomatal apertures at UUC (A), UIC (B), and 20% FA (C) amended soil inoculated with *M. incognita*.

determine the J2 nematode density in the suspension. Each millilitre of the mixture was adjusted to include 100 nematodes. At the two leaf stage of okra seedling, each pot was applied with 20 ml of nematode suspension (2000 J2). Before inoculation, three holes were gently drilled around the roots to allow the most of the J2 nematodes to reach the roots. In these holes around the seedlings roots, inoculum suspension was carefully poured. The morphological characterization of *Meloidogyne* spp. was done by observing perennial pattern arrangement (Fig. 1), following the method of Kaur and Attri [28].

2.4. Analysis of plant growth biomarkers

Plant growth characteristics like root and shoot length, root and shoot dry mass were determined. At maturity, plants were gently taken out of their pots and washed with tap water to get rid of the adhered soil. By using standard meter scale, root and shoot lengths were determined. With the assistance of electronic balance, fresh weight was taken. For dry weight, both root and shoot samples were put in a hot oven adjusted at/set to 70 °C for 48 h [29]. By employing graph paper method, leaf area (LA) was determined and leaves were counted in number.

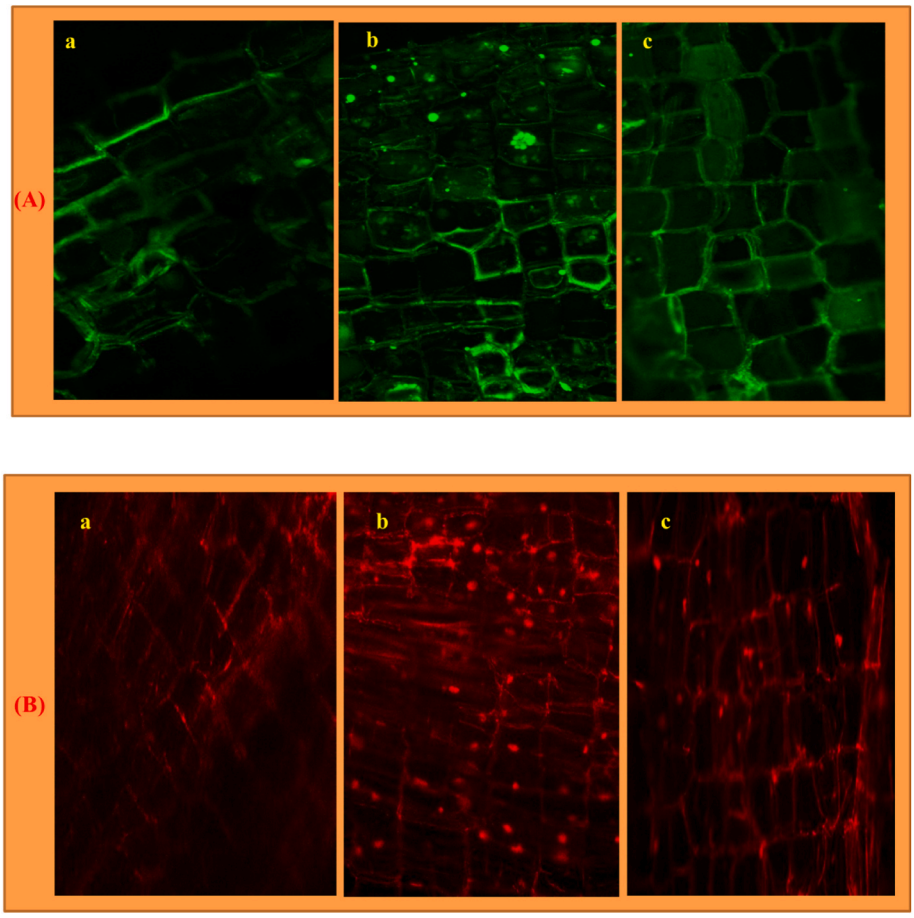


Fig. 6. ROS localization (A) and cell viability test (B). Confocal microscopic images of okra root cells obtained under UUC (a), UIC (b), and 20% FA amended soil with *M. incognita* (c).

2.5. Determination of photosynthetic pigments

Photosynthesis pigments such as chlorophyll and carotenoid content in the leaves were determined according to the MacLachalan and Zalik [30] procedure. Using a spectrophotometer, the absorbance was measured at 663 and 645 nm against a blank of 80% acetone.

2.6. Microscopic studies

2.6.1. Scanning electron microscopy

Stomatal count and their ultra-structural dimensions were found with the aid of scanning electron microscope (SEM), JEOL JSM 6510 LV, Japan. Leaves were taken and fixed with gold-palladium on SEM stubs for study [31]. Energy-dispersive X-ray spectroscopy (EDS) was used for quantification of mineral nutrients in samples.

2.6.2. Confocal microscopy

ROS and cell viability were found using fluorescent dyes called 2 7-dichlorofloresceín diacetate (DCFDA) and propidium iodide (PI) [29]. Root tips were cut off carefully and cleaned with DDW, then root tips were put in 25 μM PI solution for 10 min and placed on glass slides for observation under confocal microscope. For ROS imaging, root samples were kept in 12.5 μM DCFDA solution for 10 min then cleaned with DDW and visualized under confocal microscope.

2.7. Determination of carbohydrate and protein content

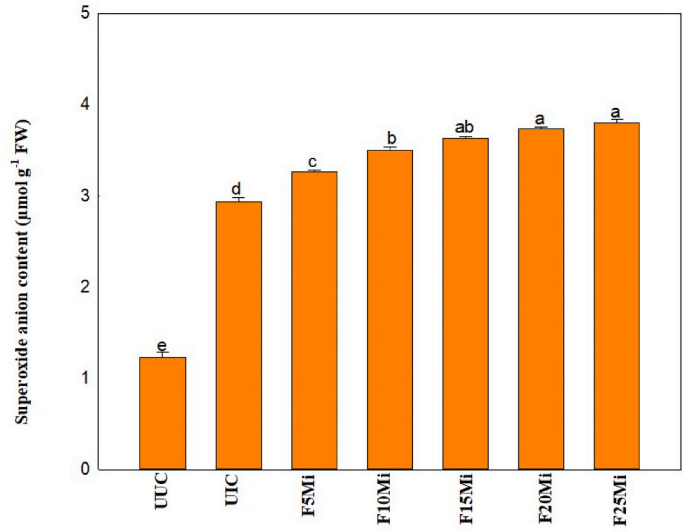
Carbohydrate content was determined by the Hedge and Hofreiter [32] assay. Leaf sample (100 mg) was homogenized in 80% ethanol and then centrifuged at 1000 rpm for 10 min. The amount of carbohydrates was then calculated from the supernatant. First, 200 μl of ethanol extract was evaporated in a water bath at 80 °C and 1 ml of ice-cold 95% sulphuric acid. Four ml of this reagent was added to the sample and heated in a boiling water bath for 8 min. The solution was then cooled and its absorbance was measured at 630 nm using spectrophotometer. D-glucose was used as a standard. Protein content was measured by Bradford [33] method in the edible part using bovine serum as a standard.

2.8. Estimation of superoxide anion ($O2^{\cdot-}$) content

The protocol of Wu et al. [34] was used to figure out the $O2^{\cdot-}$ content and its localization was carried out following the protocol of Kaur et al. [35].

2.9. Assay of antioxidant activity

Antioxidant activities were determined by homogenizing the fresh leaves using pre chilled mortar and pestle. Leaf tissue was extracted in 2 ml of 50 mM sodium phosphate buffer (pH 7.0) containing EDTA (1 mM) and polyvinylpyrolidone (0.5%). The sample was centrifuged at 13000g for 30 min, and the extract was taken for enzymatic analysis. Following a standardized procedure developed by Dhindsa et al. [36], the SOD (superoxide dismutase) activity was assessed spectrophotometrically based on the superoxide dismutase capacity to prevent the photochemical reduction of nitro blue tetrazolium. The CAT (catalase) activity was measured spectrophotometrically at 240 nm based on the consumption of H_2O_2 by using the established Beers and Sizer [37]



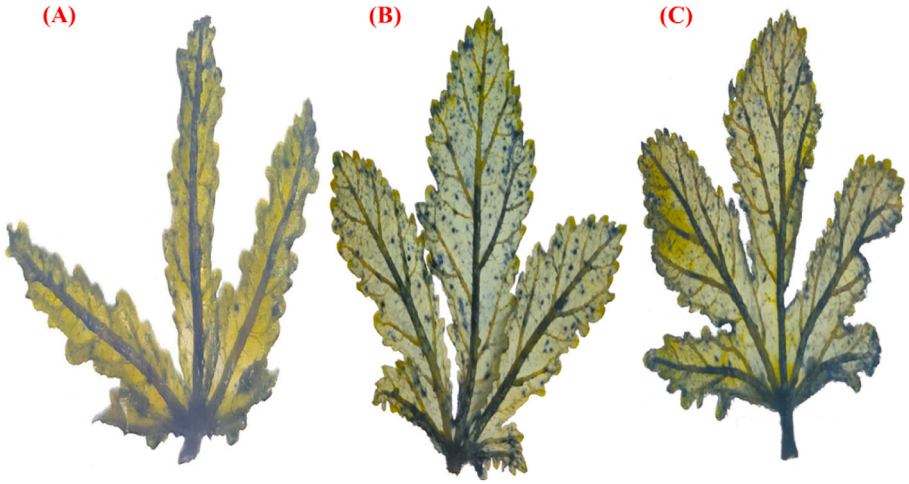


Fig. 7. Impact of various soil applied FA doses on the superoxide anion content of okra, inoculated with *M. incognita* and its histochemical localization in the leaves of okra at UUC (A), UIC (B), 20% FA amended soil with *M. incognita* (C).

methodology.

2.10. Determination of proline and lipid peroxidation

The proline content was calculated using a standardized procedure of Bates et al. [38]. Fresh leaves were homogenized in 3% sulphosalicylic acid, filtered, and then reacted with 2 ml of ninhydrin and glacial acetic acid by heating them at 100 °C. The sample was cooled in an ice bath and mixed with 4 ml of toluene, and absorbance was measured spectrophotometrically at 520 nm.

Lipid peroxidation (MDA content) was measured by using the established method of Sun et al. [39].

2.11. Disease evaluation

The crop was harvested 70 days after being inoculated. The plants were uprooted and cleaned by rinsing them under tap water to count the number of galls and egg masses. Taylor and sasser's [40] standardized procedure was used to determine the gall index (GI) and egg mass index (EMI). GI/EMI: 0 = 0 gall; 1 = 1–2 galls; 2 = 3–10 galls; 3 = 11–30 galls; 4 = 31–100 galls; 5 > 100 galls.

2.12. In-vitro studies

The effect of fly ash extracts on egg hatching and juvenile mortality of *Meloidogyne* spp. was determined by following the method of Khan et al. [41]. Different concentrations of fly ash extract were prepared by dissolving 1 kg of fly ash in 2 L of distilled water and then filtering through Whatman filter paper no. 1. The stock solution thus obtained was diluted to prepare different concentrations of extract (0%, 5%, 10%, 15%, 20%, 25%). The Coles et al. [42] formula was used to calculate percent egg hatch inhibition over control.

$$\text{Egg hatch inhibition} = \frac{No.\ of\ nematodes\ in\ control - No.\ of\ nematodes\ in\ treatment}{No.\ of\ nematodes\ in\ control} \times 100$$

Juvenile mortality of the *M. incognita* at the J2 stage was determined in different concentrations of fly ash extract (0–25%). For control, Petri dishes containing only distilled water were used. At regular interval,

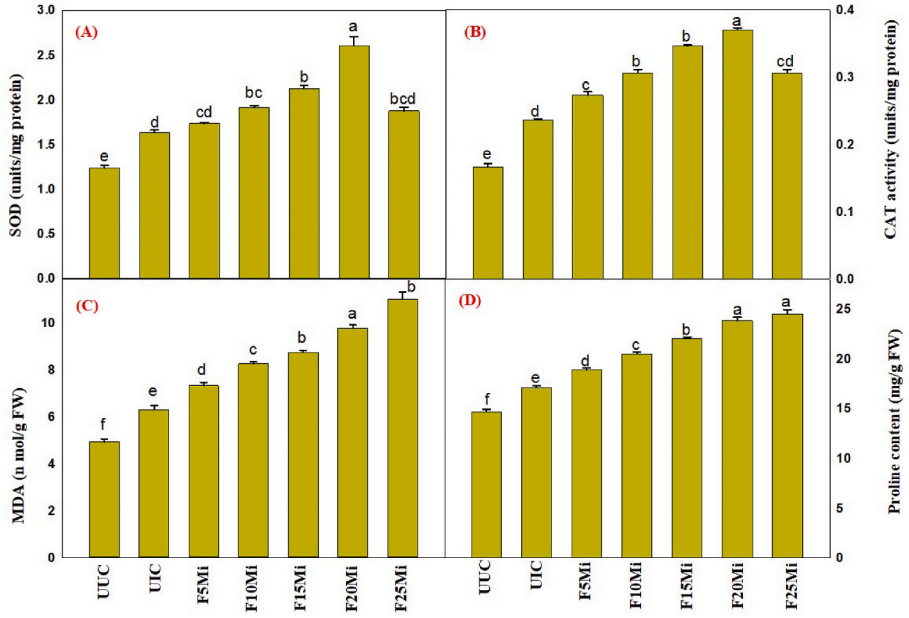


Fig. 8. Effect of various FA doses added to soil on SOD (A), CAT (B), MDA (C), and Proline content of okra inoculated with *M. incognita*.

Table 2
Effect of various FA amendments to soil on the GI, EMI, and RF of *M. incognita*.

Treatments	No. of galls per root system	Egg masses per root system	GI/ EMI	Reproduction factor
UUC	0.00	0.00	0/0	0.00
UIC	85[a]	72[a]	4/4	8[a]
F5Mi	77[b]	69[b]	4/4	7.2[ab]
F10Mi	68[c]	58[c]	4/4	6.4[b]
F15Mi	55[d]	44[d]	4/4	2.8[c]
F20Mi	28[e]	20[e]	3/3	1.4[d]
F25Mi	19[f]	10[f]	3/2	0.9[e]

dead juveniles were counted to calculate the mortality rate. The observations were taken under the stereoscopic microscope. J2 viability was further investigated by confocal microscopy using the method of Ferreira et al. [43].

$$\text{Mortality} = \frac{\text{Total no. of dead juveniles}}{\text{Total no. of nematodes}} \times 100$$

2.13. Statistical analysis

Statistical analysis was performed with the help of SPSS version 20 for windows. Standard error and analysis of variance (ANOVA) were computed using the data from 3 replicates (n = 3). Duncan's multiple range test (DMRT) was used at $P \leq 0.05$, to determine the significant differences between the means. Principal component analysis and Pearson's correlation matrix was done with the aid of origin pro (2023).

3. Results

3.1. Physiochemical properties of soil and fly ash

Energy dispersive X-ray spectroscopy (EDX) showed that FA is rich in beneficial plant nutrients like K, Mg, Ca, Fe, and Si. Amendment of 20% FA to soil improved all the essential elements except N, which is absent in FA (Table 1). The pH of fly ash was 8.5, which was more alkaline than the pH of FS, which has pH of 7.3. Fly ash particles were spherical in shape as compared to soil which were non-uniform (Fig. 2) as revealed by SEM analysis. The properties of soil improved following the addition of 20% FA. pH improved by 3.1%, EC by 76.9%, porosity by 28.7%,

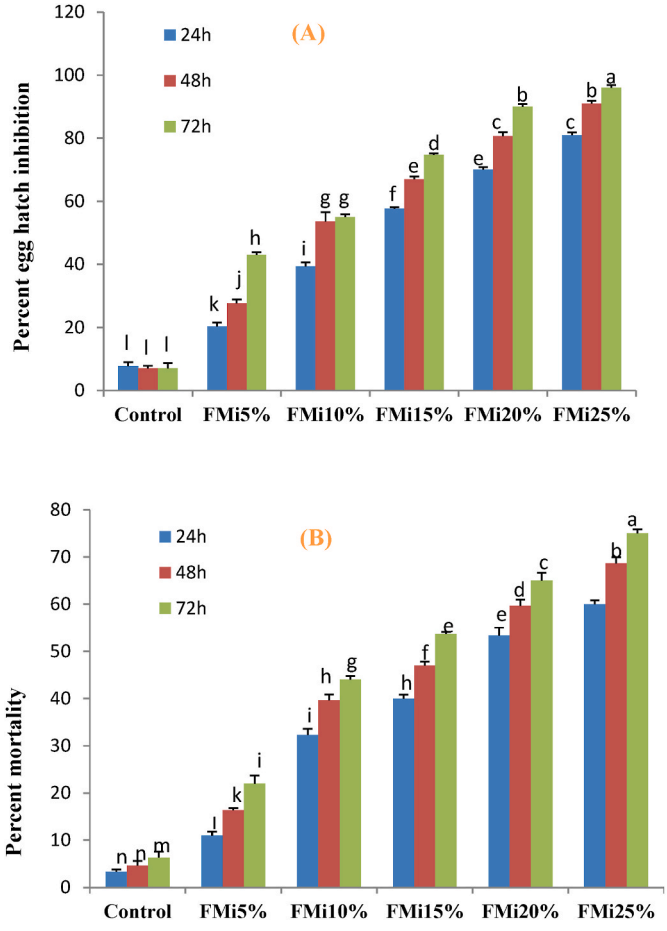


Fig. 9. *In-vitro* studies on effect of various fly ash extracts on egg hatch inhibition (A), and mortality of *M. incognita* (B).

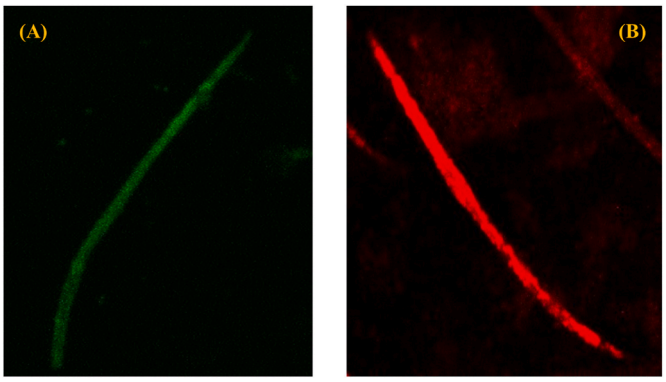


Fig. 10. Confocal microscopic images of live J2 (A), and dead J2 *M. incognita* (B).

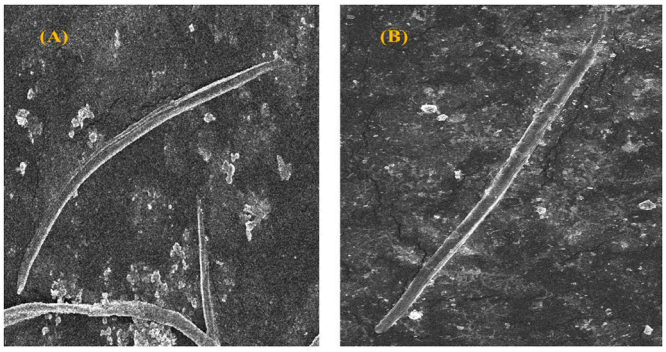


Fig. 11. SEM images of *M. incognita* at UIC (A), and 25% FA level (B).

water holding capacity by 33%, K by 29.6%, Ca by 96.2%, Cl by 45.4%, carbonate by 25.6%, bicarbonate by 70.06%, sulphate by 92.9%

3.2. Fly ash effect on plant growth, yield and pigment content of okra under nematode stress

Due to nematode inoculation, the length, fresh and dry weight of plant, and leaf number, leaf area, and stomatal area of okra dropped significantly compared to UUC (Fig. 3). The shoot length decreased by 58%, fresh and dry weight of plant by 50 and 55%, leaf number by 84%, and stomatal area by 38% as compared to UUC. Likewise, root length, also decreased by an average of 53% compared to UUC. Yield parameters of okra like total number of fruits per plant (90%), 50 seeds weight (51%), also decreased as compared to UUC. The photosynthetic pigments of okra decreased by an average of 62.2% due to *M. incognita* inoculation as compared to UUC (Fig. 4). SEM analysis demonstrated that the width of stomatal apertures decreased due to *Meloidogyne* inoculation as compared to UUC (Fig. 5)

The growth, yield, and pigment attributes of okra were considerably affected by the supplementation of fly ash (FA). Application of 20% FA significantly improved the shoot length, root length, fresh, and dry weight of plant by an average of 106.32%. No. of leaves per plant, and yield markers like fruit number per plant, fruit weight, and fruit length showed significant improvement compared to UIC (Fig. 3)

Chlorophyll contents like Ch-a, Ch-b, and carotenoids significantly increased by the treatment of 20% FA as compared to *Meloidogyne* inoculated plants (Fig. 4). Also, the width of stomatal apertures improved by the application of 20% FA as compared to UIC as revealed by SEM (Fig. 5). However, the higher doses of FA (>20%) negatively influence the growth traits of okra.

3.3. Evaluation of ROS content

ROS localization (Fig. 6A) and cell viability test (Fig. 6B) was done at 50 DAS. The level of fluorescence (ROS content) was identified by using the dye DCFDA in the root samples of okra. The intensity of fluorescence (green) was found to be higher in UIC as compared to UUC. However, at 20% FA level, fluorescence significantly reduced as compared to UIC (Fig. 6A)

3.4. Cell viability

PI was used to assess the viability of root cells. It is a nucleic acid staining dye, as it reacts with nucleic acid to signify dead cells. It was found that plants treated with 20% FA concentration showed maximum number of viable root cells, whereas *Meloidogyne incognita* inoculated plants had the highest number of dead cells (Fig. 6B).

3.5. Effect of different fly ash doses supplemented to soil on carbohydrate and protein contents of okra under nematode stress

After *M. incognita* inoculation, the biochemical markers like carbohydrate and protein declined significantly as compared to untreated uninoculated control (UUC) as shown in (Fig. 4) The impact of various fly ash doses on the biochemical properties of okra under nematode stress is shown in (Fig. 4) The supplementation of various fly ash doses to nematode infected plants improved the biochemical parameters of okra. Out of all the FA concentrations used, application of 20% fly ash was found to be most effective in improving the biochemical parameters of okra. The carbohydrate and protein contents were enhanced by an average of about 81% as compared to UIC.

3.6. Effect of FA on the superoxide anion ($O2^{\cdot-}$) in the leaves of okra under nematode stress

M. incognita inoculation substantially increased $O2^{\cdot-}$ content as compared to UUC (Fig. 7). The impact of varying fly ash levels applied to the soil on the production $O2^{\cdot-}$ in okra is shown in (Fig. 7). The concentration of $O2^{\cdot-}$ was increased by 27.2% in plants raised with the addition/supplementation of 20% FA to soil as compared to UIC. Application of higher doses of fly ash (25% or 30%), significantly increased the $O2^{\cdot-}$ levels as compared to UUC.

The localization of $O2^{\cdot-}$ in plants was depicted by histochemical staining, with $O2^{\cdot-}$ represented by blue spots (Fig. 7). The number of stained spots increases with increase in the concentrations of FA to soil inoculated with *M. incognita*.

3.7. Impact of fly ash on superoxide dismutase activity (SOD), Catalase (CAT), lipid peroxidation (MDA content), proline content

Application of varying FA doses via soil demonstrated a significant impact on the antioxidant enzymes (SOD and CAT) activities as compared to control (Fig. 8). Among the various FA levels, 20% FA level was found to be most efficient for enhancing SOD and CAT activities of okra and, consequently, lowering H_2O_2 and $O2^{\cdot-}$ build-up generated by nematode stress.

Meloidogyne incognita inoculation and different levels of FA significantly increased the MDA and proline contents of okra as compared to UIC (Fig. 8). Application of 20% FA increased the proline and MDA content of okra by 39.1 and 45.8% respectively, as compared to UIC.

3.8. Impact of different fly ash levels supplemented to soil on the gall index, egg mass index, and reproduction factor of Meloidogyne incognita on okra

The intensity of root galling and egg masses was greatest on UIC plants as compared to UUC as depicted in (Table 2), reproduction factor

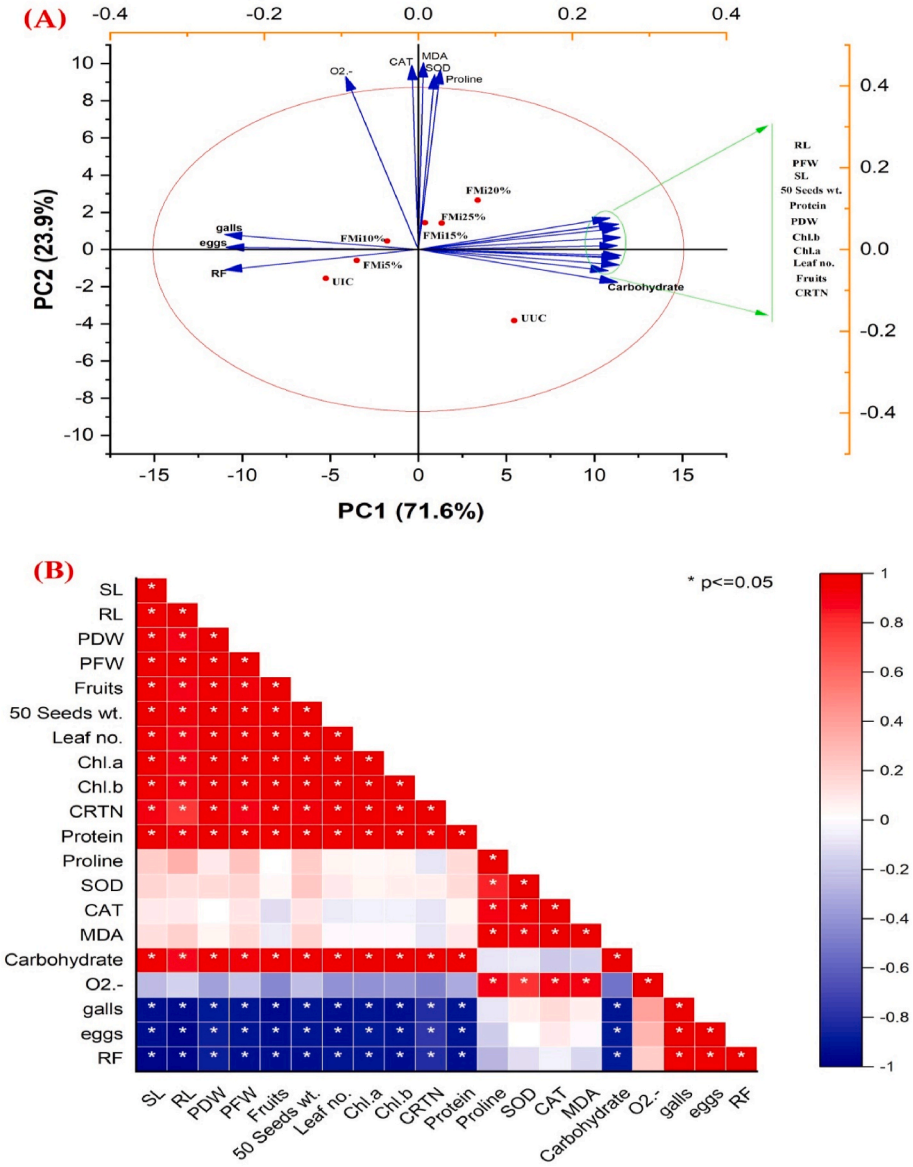


Fig. 12. Correlation analysis by principal component analysis (A) Pearson correlation matrix (B). SL = shoot length; RL = root length; PDW = plant dry weight; PFW = plant fresh weight; Chl. a = chlorophyll *a*; Chl. b = chlorophyll *b*, CRTN = carotenoids; SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde content, $O2^{\cdot-}$ = superoxide anion content; RF = reproduction factor.

of nematodes was also greatest on UIC plants. The fly ash levels (5–25%) supplemented to the soil significantly decreased the gall index (GI), egg mass index (EMI), and reproduction factor (RF) as compared to UIC. Among the various fly ash doses applied, 20% FA concentration was found to be most effective in reducing the formation of galls, and egg masses, also reproduction factor was minimum on 20% FA treated plants as compared to UIC.

3.9. In-vitro studies showing the impact of various FA levels on the hatching and mortality of Meloidogyne incognita

The impact of various fly ash extracts was observed on the hatching and mortality of *M. incognita*. Laboratory studies revealed that all the FA doses hindered the hatching of second stage juveniles (J_2) of *Meloidogyne incognita*, in a dose dependent manner as compared to untreated water, which served as control (Fig. 9A). However, maximum inhibition in hatching was observed at 25% FA extract after 72 h. Likewise, a substantial rise in mortality of second stage juveniles was observed at all the used doses of FA in a dose dependent manner as compared to control (Fig. 9B). Maximum mortality was observed at 25% FA extract after 72 h. With the help of confocal laser microscopy, contrasting fluorescent dyes, fluorescein isothiocyanate (FITC) and propidium iodide (PI), were used to selectively fix live and dead juveniles (Fig. 10). SEM images were obtained of nematodes treated with different fly ash extracts after 72 h (Fig. 11). It was found that, untreated nematodes were relatively normal, and maximum mortality was observed in nematodes treated with 25% FA extract.

4. Discussion

The management of nematodes by chemical nematicides has changed the ecosystems natural equilibrium, which has an impact on soils microbial diversity [44]. There is an urgent need for the development of efficient, sustainable, and ecologically friendly nematode control methods [45]. The properties of soil improved after the addition of fly ash. The S and CaO contents of FA are responsible for increase in soil pH. The presence of inorganic elements and metals in FA resulted in rise in EC of soil [46]. FA improves the water holding capacity of soil

through modifications to soil structure and texture [47]. Our research was supported by several studies, which demonstrated that FA can improve soils pH, EC, water holding capacity, and porosity [48,49].

FA supplementation also resulted in changes in morphology and physiology of okra plants in a dose dependent manner. *M. incognita* inoculation resulted in decrease in growth and yield of okra plants. This may possibly be due to increase in various stress biomarkers which interfere with physio-biochemical attributes and reduce photosynthetic functions. Application of 20% FA level to soil significantly improved the plant growth, yield, and pigment attributes of okra, inoculated with *M. incognita*. SEM analysis revealed that supplementation of 20% FA to soil increased the stomatal area of okra leaves (Fig. 5). The addition of vital plant nutrients like Mg, Ca, K, S, Si, P etc. to soil as well as the enhancement of soil pH and electrical conductivity could be the cause of this improvement [30,50]. Soil pH plays an important role in facilitating the availability of beneficial nutrients for plants from the soil [31]. Numerous studies have demonstrated the benefits of Silica content of FA in increasing agricultural crop yield due to its involvement in reducing the adverse effects of stress on many plant species [51]. K is essential for plant growth as it is known to activate enzymes, promotes translocation of sugars, and boost metabolism [52]. It is also crucial for opening and closing of stomata, cation-anion balance, and protein synthesis [53]. So, the observed enhancement/increment in growth and stomatal apertures of okra may be due to the addition of K ions to soil by FA. Mg plays a key role in photosynthesis as it occupies the central position in chlorophyll molecule, and acts as a cofactor for many enzymes [54]. So, due to addition of 20% FA, photosynthetic pigments increased by 128.52% with respect to UIC. Calcium being an essential element is required for plant growth and development under both stressed and non-stressed situations. It is a key component of cell wall and membrane stability and also serves as a secondary messenger in numerous physiological and developmental processes. It also increases plant's resistance to various pathogens [55]. Plant biomass production and its quality are mainly determined by iron [56]. In many biological processes like DNA synthesis, photosynthesis, and respiration iron plays an important role. For chlorophyll production in plants iron plays a crucial role and is also important for chloroplast structure and function [57]. Similar results were reported in several plants like rice (L.), wheat (L.), pumpkin (L.), and chickpea (L.) where the application of FA enhanced these parameters in a dose dependent manner [58–60].

Application of FA to soil also had an impact on protein and carbohydrate content of okra in a dose dependent manner, with 20% being the optimal dose as depicted in (Fig. 4). Increase in protein content of okra by the addition of FA is caused by the addition of K to soil, which is crucial for stimulation of enzymes needed for protein synthesis [27]. Overall, the increase in stomatal aperture, photosynthesis, electrical conductivity, pH of soil, and suppression of nematodes, all led to elevated carbohydrate and protein content. Meanwhile, Bashir et al. [61] demonstrated that highest levels of protein and carbohydrate in *Glycine max* (L.) Merr. were found in soil containing 20% FA.

Several environmental factors that affect plants may disrupt the balance between ROS production and its scavenging by antioxidant defence systems, which would result in oxidative stress [62]. ROS production occurs all the time and normally in chloroplasts during chlorophyll synthesis and electron transport of photosynthesis. Even though FA contains essential plant nutrients, it also contains some heavy metals that can cause stress on plants [58] which could damage plants by cell disintegration and damaging their lipids [63]. Our study showed that *M. incognita* inoculation and various FA levels increased the $O2^{\cdot-}$ level in okra (Fig. 7) along with significant rise in antioxidant activities (SOD and CAT) in a dose dependent manner, to scavenge the oxidative stress in order to prevent phytotoxic damage. $O2^{\cdot-}$ are mostly produced during the light reaction and they result in photoinhibition of PSI and PSII, and thereby decreasing the productivity of plants [64]. SOD catalyses the effective removal of superoxide free radicals from chloroplasts and CAT eliminates the H_2O_2 which is generated during SOD process [65]. Similar, findings were also reported in chickpea and carrot (L.) by Pandey et al. [66] and Shakeel et al. [22] respectively.

The results of the current study showed that FA application altered the MDA and proline contents of okra under nematode stress. MDA is mostly regarded as an indicator of lipid peroxidation and is a good marker of damage caused by oxidative stress [67]. In *Bacopa mannieri* (L.) Pennell, MDA content increased with *M. incognita* inoculation [68]. Proline, an osmoprotectant is a scavenger of ROS. Proline functions as a compatible solute and is essential for plants osmotic adaptations. Enhanced proline levels during stress may be due to its increased accumulation or reduced breakdown [69]. In *Sesbania cannabina* (Retz.) Pers and *Azadirachta indica* A. Juss., similar findings of increase in proline content by FA application was observed [70,71]. Our findings were also supported by Ahmad et al. [72], who reported an improvement in proline content of pumpkin under nematode stress by the application of fly ash.

In addition to the enhancement in plant growth and development, our study also revealed the nematicidal effects of fly ash. We found that FA levels applied to soil mostly reduced the GI, EMI and RF of root-knot nematodes in a dose dependent manner. The possible reason for this reduction is the presence of various essential elements in FA, which improves plants defence against nematodes [73]. Similar observations were revealed by Haris et al. [74] on carrot.

Our *in-vitro* studies, also revealed that FA levels (5–25%) significantly reduced hatching and increased the mortality of *M. incognita* when compared to UUC. The alteration in soil EC is one potential mechanism through which FA prevents hatching and increases nematode mortality [75]. Our findings were further supported by Refs. [76–78].

The relationship between the studied parameters was established by using principal component analysis (PCA) and Pearson correlation matrix (Fig. 12). The score and loading plot of PCA showed a maximum (95.5%) variation among all the parameters studied, of which PC1 contributed a 71.6% variation whereas PC2 displayed a 23.9% variation. The majority of the treatments were successfully displaced within the first two components, indicating that the application of FA had a significant ameliorative effect on all attributes when compared to untreated okra plants inoculated with *M. Incognita* (Fig. 12). PC1 was positively influenced by variables with parameters such as RL, PFW, SL, 50 seeds wt., protein, PDW, Chl-a, Chl-b, leaf no., fruits, and CRTN. Whereas, PC2 was strongly influenced by variables such as eggs, galls, and RF. PC1 and PC2 were found to have a negative correlation. SOD, CAT, proline, $O2^{\cdot-}$, and MDA, on the other hand, were contributed by both PC1 and PC2. In the case of the score plot, the 20% FA treatment had the greatest addition to PC1, which has a strong negative correlation to UIC. Furthermore, the 15%, 25% FA treatments showed a positive correlation with PC1. In contrast, the treatments with 5% and 10% FA had no significant ameliorative effect on the nematode treated plants and contributed the most to PC2. In general, when compared to lower doses of FA, the concentration of FA (20%) significantly reduced the stress responses induced by nematodes and proved most effective in overcoming the negative responses induced by the inoculation of *M. incognita*.

5. Conclusion

Fly ash has a considerable potential in agriculture due to its effectiveness in improving soil conditions, crop yield, and also in reducing the industrial waste. Our results showed that FA elicited the morpho-physiology and biochemistry of okra plants in a dose dependent manner. Fly ash levels (5–20%) had a positive effect on okra by increasing plant tolerance, and acts on photosynthetic and antioxidant machinery to promote growth of okra and higher doses (>20%) showed negative impact on okra, by impairing various physio-biochemical, microscopic, and histochemical attributes. The gall index, egg mass index, and reproduction factor of *M. incognita* were significantly reduced

by fly ash concentrations in a dose dependent manner. Therefore, optimum doses of fly ash not only promote plant growth and yield, but also suppress the root-knot nematodes. Further research needs to be done to confirm the present studies at molecular level and the pathways altered by fly ash, so that it can be recommended to farmers.

Author contributions

Adil ameen bhat: Data collection, Methodology, Analysis, Prepared original draft. **Adnan shakeel:** Methodology, Preparation and interpretation of manuscript. **Hosny H. Kesba** and **Zafar Ahmad Handoo:** Manuscript design. **Abrar Ahmed Khan**: Conceptualization, Supervision and interpretation of manuscript. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors state that they have no known financial or personal relationships that could be seen to have influenced the work reported in this paper.

Data availability

Data will be made available on request.

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